Canonical NF- B signaling in hepatocytes acts as a tumor-suppressor in hepatitis B virus surface antigen-driven hepatocellular carcinoma by controlling the unfolded protein response

Sunami, Yoshiaki ; Ringelhan, Marc ; Kokai, Enikö ; Lu, Miao ; O'Connor, Tracy ; Lorentzen, Anna ; Weber, Achim ; Rodewald, Ann-Katrin ; Müllhaupt, Beat ; Terracciano, Luigi ; Gul, Sarah ; Wissel, Sebastian ; Leithäuser, Frank ; Krappmann, Daniel ; Riedl, Petra ; Hartmann, Daniel ; Schirmbeck, Reinhold ; Strnad, Pavel ; Hüser, Norbert ; Kleeff, Jörg ; Friess, Helmut ; Schmid, Roland M ; Geisler, Fabian ; Wirth, Thomas ; Heikenwalder, Mathias

Abstract: UNLABELLED Chronic hepatitis B virus (HBV) infection remains the most common risk factor for hepatocellular carcinoma (HCC). Efficient suppression of HBV viremia and necroinflammation as a result of nucleos(t)ide analogue treatment is able to reduce HCC incidence; nevertheless, hepatocarcinogenesis can occur in the absence of active hepatitis, correlating with high HBV surface antigen (HBsAg) levels. Nuclear factor B (NF- B) is a central player in chronic inflammation and HCC development. However, in the absence of severe chronic inflammation, the role of NF- B signaling in HCC development remains elusive. As a model of hepatocarcinogenesis driven by accumulation of HBV envelope polypeptides, HBsAg transgenic mice, which show no HBV-specific immune response, were crossed to animals with hepatocyte-specific inhibition of canonical NF- B signaling. We detected prolonged, severe endoplasmic reticulum stress already at 20 weeks of age in NF- B-deficient hepatocytes of HBsAg-expressing mice. The unfolded protein response regulator binding immunoglobulin protein/78-kDa glucose-regulated protein was down-regulated, activating transcription factor 6, and eIF2 were activated with subsequent overexpression of CCAAT/enhancer binding protein homologous protein. Notably, immune cell infiltrates and liver transaminases were unchanged. However, as a result of this increased cellular stress, insufficient hepatocyte proliferation due to G1 /S-phase cell cycle arrest with overexpression of p27 and emergence of ductular reactions was detected. This culminated in increased DNA damage already at 20 weeks of age and finally led to 100% HCC incidence due to NF- B inhibition. CONCLUSION The role of canonical NF- B signaling in HCC development depends on the mode of liver damage; in the case of HBsAg-driven hepatocarcinogenesis, NF- B in hepatocytes acts as a critical tumor suppressor by augmenting the endoplasmic reticulum stress response. (Hepatology 2016;63:1592-1607).

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Canonical NF-κB Signaling in Hepatocytes Acts as a Tumor-Suppressor in Hepatitis B Virus Surface Antigen-Driven Hepatocellular Carcinoma by Controlling the Unfolded Protein Response

Yoshiaki Sunami,[1,2*] Marc Ringelhan,[3–5*] Enikö Kokai,[2] Miao Lu,[1] Tracy O'Connor,[4] Anna Lorentzen,[4] Achim Weber,[6] Ann-Katrin Rodewald,[6] Beat Müllhaupt,[7] Luigi Terracciano,[8] Sarah Gul,[2] Sebastian Wissel,[2] Frank Leithäuser,[9] Daniel Krappmann,[10] Petra Riedl,[11] Daniel Hartmann,[1] Reinhold Schirmbeck,[11] Pavel Strnad,[12] Norbert Hüser,[1] Jörg Kleeff,[1] Helmut Friess,[1] Roland M Schmid,[3] Fabian Geisler,[3] Thomas Wirth,[2*] and Mathias Heikenwalder[4,13*]

Chronic hepatitis B virus (HBV) infection remains the most common risk factor for hepatocellular carcinoma (HCC). Efficient suppression of HBV viremia and necroinflammation as a result of nucleos(t)ide analogue treatment is able to reduce HCC incidence; nevertheless, hepatocarcinogenesis can occur in the absence of active hepatitis, correlating with high HBV surface antigen (HBsAg) levels. Nuclear factor κB (NF-κB) is a central player in chronic inflammation and HCC development. However, in the absence of severe chronic inflammation, the role of NF-κB signaling in HCC development remains elusive. As a model of hepatocarcinogenesis driven by accumulation of HBV envelope polypeptides, HBsAg transgenic mice, which show no HBV-specific immune response, were crossed to animals with hepatocyte-specific inhibition of canonical NF-κB signaling. We detected prolonged, severe endoplasmic reticulum stress already at 20 weeks of age in NF-κB-deficient hepatocytes of HBsAg-expressing mice. The unfolded protein response regulator binding immunoglobulin protein/78-kDa glucose-regulated protein was down-regulated, activating transcription factor 6, and eIF2α were activated with subsequent overexpression of CCAAT/enhancer binding protein homologous protein. Notably, immune cell infiltrates and liver transaminases were unchanged. However, as a result of this increased cellular stress, insufficient hepatocyte proliferation due to G_1/S-phase cell cycle arrest with overexpression of p27 and emergence of ductular reactions was detected. This culminated in increased DNA damage already at 20 weeks of age and finally led to 100% HCC incidence due to NF-κB inhibition. *Conclusion:* The role of canonical NF-κB signaling in HCC development depends on the mode of liver damage; in the case of HBsAg-driven hepatocarcinogenesis, NF-κB in hepatocytes acts as a critical tumor suppressor by augmenting the endoplasmic reticulum stress response. (HEPATOLOGY 2016;63:1592-1607)

Abbreviations: ATF, activating transcription factor; BiP, binding immunoglobulin protein; CHB, chronic hepatitis B; CHOP, CCAAT/enhancer binding protein homologous protein; ER, endoplasmic reticulum; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IHC, immunohistochemical; IKK, IκB kinase; IL-6, interleukin-6; mRNA, messenger RNA; NF-κB, nuclear factor κB; qPCR, quantitative polymerase chain reaction; TNF, tumor necrosis factor; UPR, unfolded protein response.

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Chronic hepatitis B viral (HBV) infection is the most common chronic viral infection in the world and, besides end-stage liver disease, causes hepatocarcinogenesis, accounting for more than half of all hepatocellular carcinoma (HCC) cases.[1] HCC is the fifth most common type of cancer and the second most common cause of cancer-related death worldwide.[2] Many patients are not eligible for curative surgical resection or liver transplantation, and chances for curative treatment options for this chemoresistant tumor remain low.

In hepatocarcinogenesis a sequence of chronic inflammation, cell death, compensatory liver proliferation, and subsequent liver cirrhosis as well as altered cytokine networks and signaling pathways, including nuclear factor κB (NF-κB) signaling, are well-established oncogenic factors.[3-5]

It was previously shown that in HBV-induced or hepatitis C virus (HCV)-induced hepatitis and subsequent inflammation-driven hepatocellular carcinogenesis, NF-κB signaling is activated[3,4] and causally linked to the development of HCC.[5]

In persistent HBV infections, mainly an ineffective T-cell response causes repeated liver damage.[6] Intriguingly, HCC develops in 10%-20% of patients chronically infected with HBV in the absence of cirrhosis and severe necroinflammation, which is in contrast to most other HCC etiologies.[7] Treatment with nucleos(t)ide analogues that control HBV replication in chronic hepatitis B (CHB) infection greatly reduces necroinflammation and subsequent risk of HCC development but does not eliminate the risk for HCC, regardless of the presence of baseline liver cirrhosis.[8] Despite efficient suppression of HBV viremia as a result of long-term nucleos(t)ide analogue therapy, hepatocarcinogenesis is still observed, especially in patients with high HBV surface-antigen (HBsAg) levels.[9,10] Furthermore, patients with spontaneous HBV DNA clearance and residual HBsAg titers >1000 IU/mL show increased HCC risk.[11] This is due to well-established direct oncogenic effects of HBV, including HBV DNA integration and multiple effects of the enigmatic HBV x-protein.[8,12] In addition, almost three decades ago, the typical histological finding of "ground glass" hepatocytes showing accumulation of HBV-surface proteins first demonstrated the direct oncogenic potential of HBsAgs in particular situations.[13,14] Similarly, accumulation of HBV surface proteins, overexpression of HBV large surface proteins, preS1/2 mutants, and C-terminally truncated M protein can be frequently found in humans

S. Wissel's current address is: Institute of Molecular Biotechnology, Vienna, Austria

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ARTICLE INFORMATION:

From the [1]Department of General Surgery, Klinikum rechts der Isar, Technical University Munich, Munich, Germany; [2]Institute of Physiological Chemistry, University of Ulm, Ulm, Germany; [3]Department of Internal Medicine II, Klinikum rechts der Isar, Technical University Munich, Munich, Germany; [4]Institute of Virology, Technical University Munich/Helmholtz Zentrum Munich, Munich, Germany; [5]German Center for Infection research (DZIF), Munich partner site, Munich, Germany; [6]Institute of Surgical Pathology, Zürich, Switzerland; [7]Department of Gastroenterology and Hepatology, University Hospital Zürich, Zürich, Switzerland; [8]Institute of Pathology, University Hospital Basel, Basel, Switzerland; [9]Institute of Pathology, University Medical Center Ulm, Ulm, Germany; [10]Research Unit Cellular Signal Integration, Helmholtz Zentrum Munich, Munich, Germany; [11]Department of Internal Medicine I, University Medical Center Ulm, Ulm, Germany; [12]Department of Medicine III and IZKF, University Hospital Aachen, Aachen, Germany; [13]Division of Chronic Inflammation and Cancer, German Cancer Research Center (DKFZ), Heidelberg, Germany.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Mathias Heikenwalder
Institute of Virology
Technical University Munich
Schneckenburgerstrasse 8, 81675 Munich, Germany
E-mail: heikenwaelder@helmholtz-muenchen.de
tel: +49 89-4140 7440
fax: +49 89-4140 7444
or

Thomas Wirth
Institute of Physiological Chemistry
University of Ulm
Albert-Einstein-Allee 11, 89081 Ulm, Germany
E-mail: thomas.wirth@uni-ulm.de
tel: +49 731-500 23270
fax: +49 731-500 22892

and exert oncogenic functions.[15-17] Accumulation of HBV surface proteins and especially large HBV surface proteins in the endoplasmic reticulum (ER) has been shown to induce ER stress and the unfolded protein response (UPR) and to activate NF-κB.[13,14,18] The UPR is activated in response to ER accumulation of (mainly) unfolded or misfolded proteins. Activation of the UPR eliminates misfolded proteins through ER-associated degradation, inhibition of protein synthesis,[19] or initiation of apoptosis when ER stress is sustained or too severe. Sustained UPR is known to be oncogenic, inducing DNA damage and interfering with cell proliferation, survival, and the cell cycle.[18] A key regulator of ER stress-associated apoptosis is CCAAT/enhancer binding protein homologous protein (CHOP), also known as growth arrest and DNA damage-inducible gene 153; and unalleviated ER stress responses induce tumor formation.[20]

Most importantly, observations in patients with CHB under conditions of immune suppression suggest that in the absence of adaptive immune responses, cellular stress induced by HBV may also lead to development of liver disease and carcinogenesis.[21-23]

The transcription factor NF-κB and its upstream kinase complex IκB kinase 1 (IKK1)/IKKα, IKK2/IKKβ, and NEMO/IKKγ are master regulators of cellular proliferation, apoptosis, and inflammation which also play a central role in hepatocarcinogenesis.[24] Several studies demonstrate that the NF-κB system is deregulated due to viral infection and is associated with tumor development,[25,26] and the link between NF-κB activation, ER stress, and liver damage is well established.[19,27-29] Yet divergent roles of NF-κB in hepatocarcinogenesis have been reported, depending on the HCC model used.[24,30]

Inhibition of NF-κB signaling in hepatocytes attenuates inflammation-associated HCC,[3] and hepatic NF-κB signaling activated by lymphotoxins α and β plays a key role in driving both virally and metabolically associated hepatocarcinogenesis.[4,31] On the other hand, liver-specific inhibition of NF-κB signaling by conditional deletion of IKK2 promotes carcinogen (diethylnitrosamine)-induced liver cancer.[5]

Therefore, we speculated that NF-κB signaling plays a crucial role in hepatocytes expressing high levels of HBsAg in the absence of specific inflammation due to central immune tolerance toward the transgene. To test this theory and to determine its role in ER stress-driven HCC development, we ablated canonical NF-κB signaling specifically in hepatocytes of HBsAg-overexpressing transgenic mice.

Materials and Methods

HUMAN SAMPLES

Human liver samples from patients with chronic HBV (with or without HCC, inactive and active carrier state), with chronic hepatitis C viral infection, or with nonalcoholic steatohepatitis and healthy liver samples were obtained from the Department of General Surgery, MRI, TU-Munich (Munich, Germany), Institute of Surgical Pathology (Zürich, Switzerland), and Institute of Pathology (Basel, Switzerland). The project was authorized by the ethics committee at the TU-Munich (ref: 5846/13), the University of Zürich (ref: StV26/2005,KEK-ZH-Nr. 2013-0382), and the University Hospital Basel (ref: EKBB,Nr 313/13).

ANIMAL STUDIES

Transgenic mice expressing HBsAg (Tg[Alb-lHBV]Bri44) overexpress all HBV envelope proteins and have been described.[13,14]

In order to inhibit canonical NF-κB signaling in hepatocytes, we crossed transgenic mice carrying a tetracycline-inducible dominant-negative human IKK2 (IKK2KD) allele under the control of a bidirectional promoter with animals expressing tetracycline-responsive transactivator (TET Systems) under the control of the rat liver activator protein promoter.[32] All mice were on a C57BL/6 and NMRI mixed background, and resulting heterozygous $HBsAg^{+}$/$IKK2KD^{Hep}$ mice, $HBsAg^{+}$ single transgenic littermates, and transgenic negative control male mice were used in this study unless otherwise specified. Mice were kept under specific pathogen-free conditions. All mice received 0.1 g/L doxycycline in the drinking water until 3 weeks of age (also see Supporting Information).

IMMUNOHISTOCHEMICAL ANALYSIS

Histological analysis with 2 μm paraffin sections was performed with the Bond Polymer Refine Detection Kit (Leica) on the BondMax system (Leica).

For quantification of stainings, whole slides were scanned using an SCN400 slide scanner (Leica) and analyzed using Tissue IA image analysis software (Leica) with optimized quantification algorithms. For quantification, two or three random 8-15 mm^2 areas of liver tissue on each slide were chosen, and obtained

values were merged for further statistical analysis (also see Supporting Information).

GENE EXPRESSION ANALYSIS AND 16-GENE ANALYSIS

Two step real-time quantitative polymerase chain reaction (qPCR) from whole liver/tumor tissue was carried out using SYBR green on a Fast Real Time PCR System (Applied Biosystems). A 16-gene analysis using qPCR for characterizing liver tumors according to their degree of proliferation and differentiation was performed as described.[31] All real-time PCRs were performed at least in duplicate. All values were normalized to the level of *Gapdh* (or *Rhot2* in 16-gene analysis) messenger RNA (mRNA; change in cycle threshold). The mean change in cycle threshold values from age-matched controls was used to calculate fold change.

WESTERN BLOTTING AND ELECTROPHORETIC MOBILITY SHIFT ASSAY

Western blotting and electrophoretic mobility shift assay experiments were performed as described.[32] For antibodies used, see Supporting Information.

HBsAg SERUM ANALYSIS

HBsAg was measured in mouse sera (1:50 dilution) on an Architect (Abbott) using a validated two-step HBsAg immunoassay (chemiluminescent microparticle immunoassay) for the qualitative and quantitative detection of HBsAg.

HSPA5 AND HSPA5 PROMOTER ANALYSIS

RelA sites (model ID MA0107.1) were analyzed using JASPAR with a relative profile score threshold of 85% for the HSPA5 (ENSG00000044574) and hspa5 (ENSMUSG00000026864) genes with a 5′ flanking region of 1000 base pairs; gene structure and predicted sites were visualized using the Integrative Genomics Viewer (also see Supporting Information).

STATISTICAL ANALYSIS

Statistical analysis was performed with GraphPad Prism software, version 6.0 (GraphPad Software). Data are shown as means ± standard error of the mean, if not indicated differently. Statistical significance was determined using a two-tailed Student *t* test. $P < 0.05$ was considered significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Results

CANONICAL NF-κB SIGNALING IS ACTIVATED IN LIVER PARENCHYMAL CELLS OF CHRONIC HBV PATIENTS AND MICE EXPRESSING HBsAg

In patients chronically infected with HBV, including in inactive carriers (hepatitis B e antigen-negative, showing no significant necroinflammation, and HBV DNA <2000 IU/mL), we observed nuclear translocation of RelA/p65 (Fig. 1A and Supporting Table S1). Nuclear translocation of RelA/p65 correlated with grade of inflammatory activity in CHB, hepatitis C, and nonalcoholic steatohepatitis (Fig. 1B; Supporting Fig. S1A,B); however, also inactive HBV carriers show RelA/p65^{+} hepatocytes mainly in HBsAg-positive areas (Fig. 1A). Thus, we asked whether NF-κB signaling could play an oncogenic or protective role in hepatocytes of chronically HBV-infected patients who show no overt inflammation but express viral transcripts at a high level.

In order to determine the role of canonical NF-κB signaling in disease onset and HCC development due to high expression and accumulation of HBsAgs in the ER, we used the well-characterized transgenic mouse model exhibiting hepatocyte-specific expression of L, M, and S HBV surface proteins at high levels in the absence of specific inflammation (Fig. 1C), referred to as HBsAg^{+} animals.[13,14] These mice show liver cell injury by 4 months and HCC (with an incidence of approximately 50%) by 12-20 months due to accumulation of HBV surface proteins.[13,14] Gradually increasing liver damage over time was evident in HBsAg^{+} mice, with significantly elevated liver transaminase levels (Fig. 1D). Interestingly, in HBsAg^{+} transgenic animals we detected RelA/p65-positive hepatocyte nuclei, similar to human inactive CHB samples (Fig. 1C; Supporting Fig. S2E-G). Activation of canonical NF-κB signaling was confirmed by electrophoretic mobility shift assay, which showed enhanced NF-κB DNA-binding activity in whole-liver lysates of HBsAg^{+} transgenics (Fig. 1E).

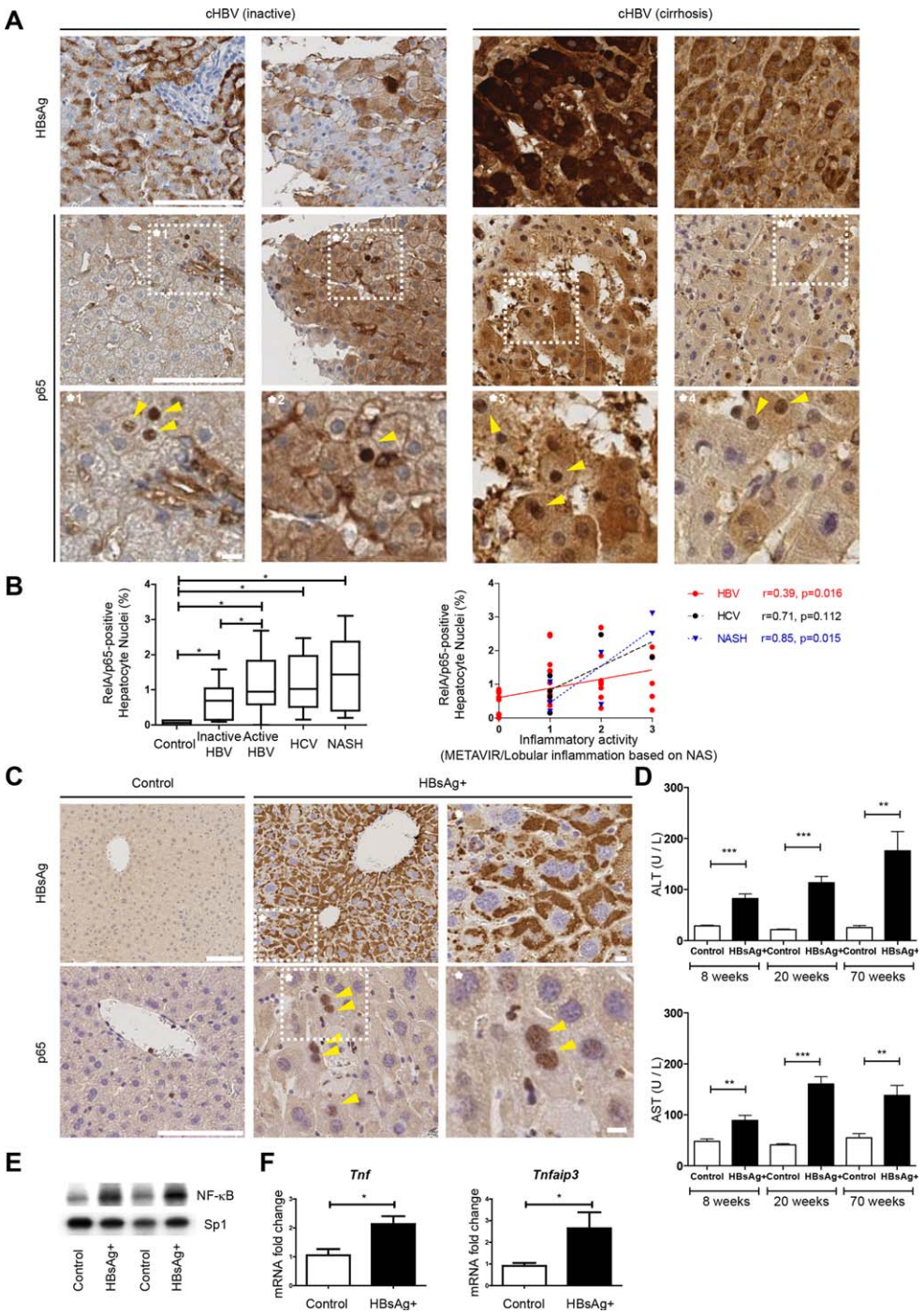

FIG. 1. Activated NF-κB signaling in CHB and HBsAg overexpression. (A) Detection of activated NF-κB signaling in hepatocytes of chronically HBV-infected patients especially in areas of HBsAg expression, as determined by IHC for HBsAg and RelA/p65. This finding was also apparent in patients with inactive CHB (HBV DNA <2000 IU/mL, no significant necroinflammation [METAVIR ≤A1], hepatitis B e antigen negative; bar = 100 μm). Lower panel shows magnification of RelA/p65-IHC (yellow arrows indicating RelA/p65-positive hepatocytes; bar = 10 μm). (B) Quantification of RelA/p65-positive hepatocyte nuclei as a percentage of total hepatocyte nuclei in patients with "inactive" and "active" hepatitis B as well as patients chronically infected with HCV, patients with nonalcoholic steatohepatitis, and healthy liver tissue as control (left graph). Positive correlation of histological inflammatory stage (METAVIR 0-3)/nonalcoholic steatohepatitis grading for lobular inflammation (based on part of nonalcoholic fatty liver disease activity score 0-3) with finding of RelA/p65-positive hepatocyte nuclei (percentage) (right graph; *r* and *p* values are shown in legend). (C) HBsAg expression and activation of NF-κB in hepatocytes of HBsAg$^+$ transgenic mice shown by IHC for HBsAg and RelA/p65 nuclear translocation (yellow arrows; bar = 100 μm) and magnification (bar = 10 μm). (D) Alanine aminotransferase and aspartate aminotransferase levels were elevated in 8-week-old, 20-week-old, and 70-week-old HBsAg$^+$ versus control mice (control n = 10-26, HBsAg$^+$ n = 13-28). (E) NF-κB DNA-binding activity was proven by electrophoretic mobility shift assay in liver extracts from 20-week-old control and HBsAg$^+$ mice. Sp1 electrophoretic mobility shift assay was performed as a control. (F) Expression of NF-κB target genes TNFα and A20 determined by qPCR was up-regulated in livers from HBsAg$^+$ mice (fold change; TNFα n = 4, A20 n = 6). Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis.

Moreover, mRNA expression of NF-κB target genes such as *Tnfa* (tumor necrosis factor-α [TNFα]) and *Tnfaip3* (A20) was significantly up-regulated in livers of $HBsAg^+$ livers (Fig. 1F). These data suggest that overexpression of HBsAgs alone is sufficient to induce canonical NF-κB activation in hepatocytes. This is consistent with our finding of canonical NF-κB activation in hepatocytes of patients expressing HBsAg with inactive CHB and with previously reported activation of canonical NF-κB signaling in CHB and subsequent HCC development.[3-5]

HEPATOCYTE-SPECIFIC INHIBITION OF CANONICAL NF-κB INCREASES HEPATOCELLULAR CARCINOGENESIS IN HBsAg TRANSGENIC MICE

To determine whether NF-κB signaling influences HCC formation in HBsAg transgenic livers, we crossed $HBsAg^+$ transgenic mice to mice expressing a dominant negative form of IKK2/IKKβ (IKK2KD) in hepatocytes, resulting in inhibition of canonical NF-κB signaling ($IKK2KD^{Hep}$ mice). Transgenic dominant negative IKK2 expression was repressed by doxycycline administration in the drinking water until the age of 3 weeks to rule out effects in liver organogenesis. Removal of doxycycline at that time point led to liver-specific induction of a luciferase reporter gene as well as IKK2KD expression (Supporting Fig. S2A-C). Activation of canonical NF-κB signaling in whole-liver lysates of $HBsAg^+/IKK2KD^{Hep}$ double-transgenic mice was decreased compared to $HBsAg^+$ as shown by phosphorylated RelA/p65 western blot (Supporting Fig. S2C,D). No $RelA/p65^+$ hepatocyte nuclei were detectable after inhibition of canonical NF-κB in $HBsAg^+/IKK2KD^{Hep}$ mice (Supporting Fig. S2E-G), and mRNA expression of NF-κB target genes such as *Tnfa*, *Tnfaip3*, *Tnfsf14*, and *Tnfsf10* was significantly down-regulated compared to $HBsAg^+$ and control animals (Supporting Fig. S2H).

Of note, inhibition of canonical NF-κB signaling in $HBsAg^+/IKK2KD^{Hep}$ mice induced a dramatic increase in tumor incidence, number, and size: HCC of $HBsAg^+/IKK2KD^{Hep}$ mice was characterized by large, macroscopically visible tumors compared to $HBsAg^+$ single-transgenic animals which displayed small tumor nodules (Fig. 2A). Tumor incidence was increased to 100% in $HBsAg^+/IKK2KD^{Hep}$ mice compared to ~50% in HBsAg single-transgenic mice at 70 weeks (Fig. 2B). Furthermore, $HBsAg^+/IKK2KD^{Hep}$ mice displayed a significantly higher number of tumor nodules and a larger maximum tumor size (Fig. 2B). These findings were consistent with an overall increase in liver weight and liver transaminase levels (alanine aminotransferase and aspartate aminotransferase) (Fig. 2C).

A thorough histological analysis of all tumors showed several histological HCC subtypes in both $HBsAg^+/IKK2KD^{Hep}$ and $HBsAg^+$ mice (Fig. 2D; Supporting Fig. S3B). RNA expression analysis of proliferation and differentiation genes of tumor nodules was performed and compared to control tissue of semimalignant HCC of WHV/N-Myc2 transgenic p53 heterozygous and poorly differentiated tumors of WHV/N-Myc2 transgenic p53 knockout. HCC from $HBsAg^+/IKK2KD^{Hep}$ mice frequently showed alpha-fetoprotein expression and a more malignant phenotype in a 16-gene analysis (Fig. 2E). However, there was no significant difference in the number of $Ki67^+$ cells in HCC between genotypes (Supporting Fig. S3C). Of note, analysis of 70-week-old $IKK2KD^{Hep}$ single-transgenic mice revealed small adenomas in one of six animals (Supporting Fig. S3D).

These data suggest that canonical NF-κB signaling is a crucial antitumorigenic factor in HCC development in the context of high HBsAg expression, which is in contrast to HCC that arises in the context of a chronic inflammatory environment.[3,4]

INHIBITED CANONICAL NF-κB SIGNALING IN $HBsAg^+$ TRANSGENIC MICE DISRUPTS COMPENSATORY LIVER REGENERATION

Regarding the observed tumor-suppressive role of NF-κB, we investigated whether NF-κB inhibition in $HBsAg^+$ mice enhances secondary inflammatory responses in mice at 20 weeks (i.e., preneoplastic). However, immunohistological staining for $CD3^+$ (T lymphocytes) and $F4/80^+$ (macrophages, Kupffer cells) revealed no significant change in invading inflammatory cells in $HBsAg^+/IKK2KD^{Hep}$ transgenic mice compared to $HBsAg^+$ transgenic mice (Fig. 3A), nor did we detect a significant increase in expression of the inflammatory cytokine interleukin-6 (IL-6) in $HBsAg^+$, $HBsAg^+/IKK2KD^{Hep}$, or control livers by qPCR or enzyme-linked immunosorbent assay (Fig.

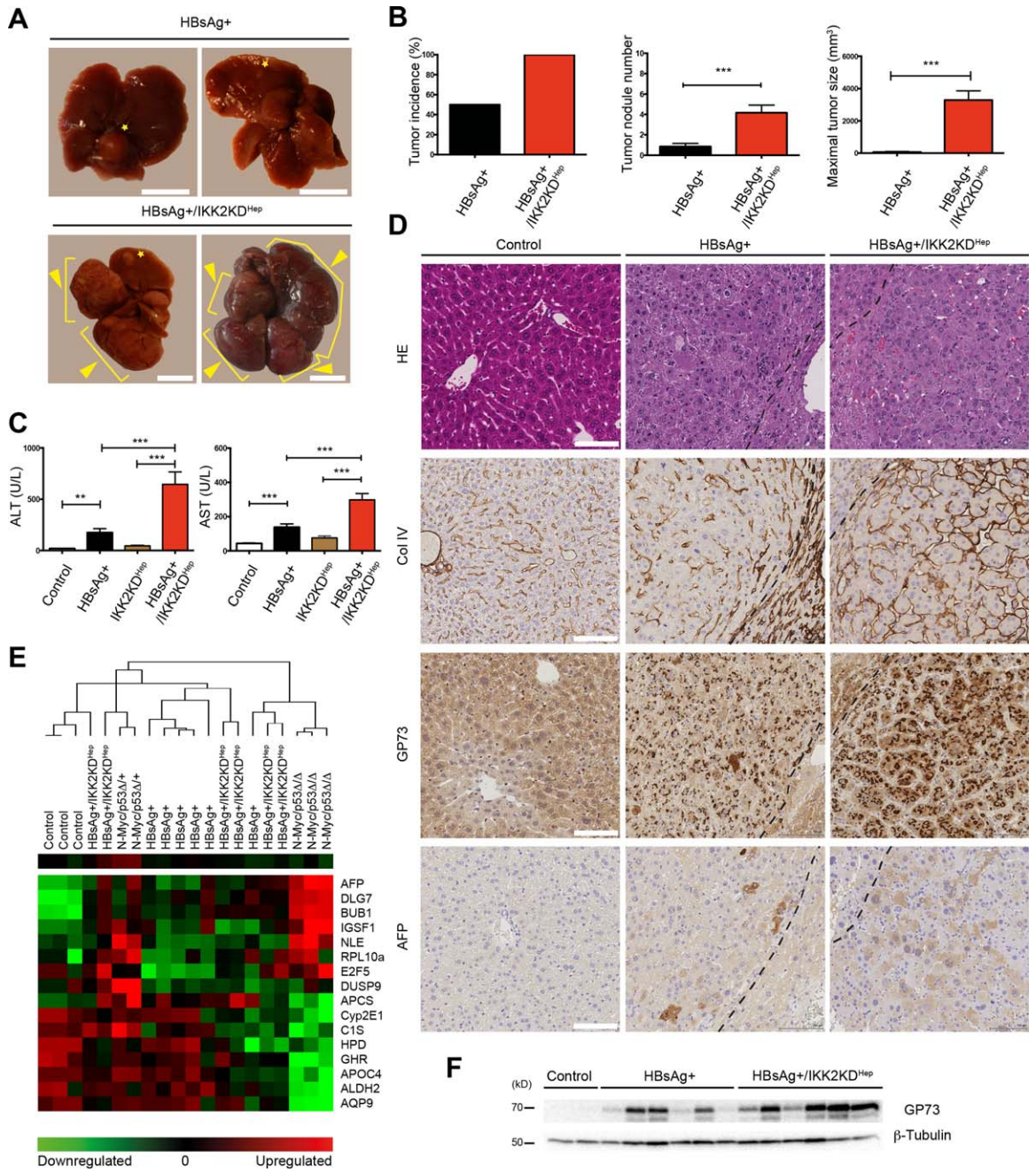

FIG. 2. Expression of a dominant-negative form of IKK2 in the liver leads to enhanced HBsAg-induced tumorigenesis. (A) Macroscopic appearance of liver tumors in 70-week-old mice. *Small tumor nodules. Brackets and yellow arrows indicate massive tumors in $HBsAg^{+}$/$IKK2KD^{Hep}$ mice (bar = 1 cm). (B) Incidence of tumors (>1 mm) in 70-week-old mice, number of macroscopically apparent tumor nodules (>1 mm), and maximal macroscopic tumor volume ($HBsAg^{+}$ n = 13, $HBsAg^{+}$/$IKK2KD^{Hep}$ n = 6). (C) Alanine aminotransferase and aspartate aminotransferase levels measured in 70-week-old mice (n = 8-14). (D) Representative hematoxylin and eosin and IHC staining for collagen IV, GP73 (golph2), and alpha-fetoprotein displaying typical HCC features (bar = 100 μm; dotted line marks HCC boarder). (E) Sixteen-gene analysis by qPCR of tumors derived from $HBsAg^{+}$ and $HBsAg^{+}$/$IKK2KD^{Hep}$ mice compared to semimalignant HCC from WHV/N-Myc2tg/$p53^{+}/\Delta$ mice and poorly differentiated tumors from WHV/N-Myc2/p53Δ/Δ mice. (F) Western blot analysis for GP73 and β-tubulin as loading control. Abbreviations: AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Col IV, collagen IV; HE, hematoxylin and eosin.

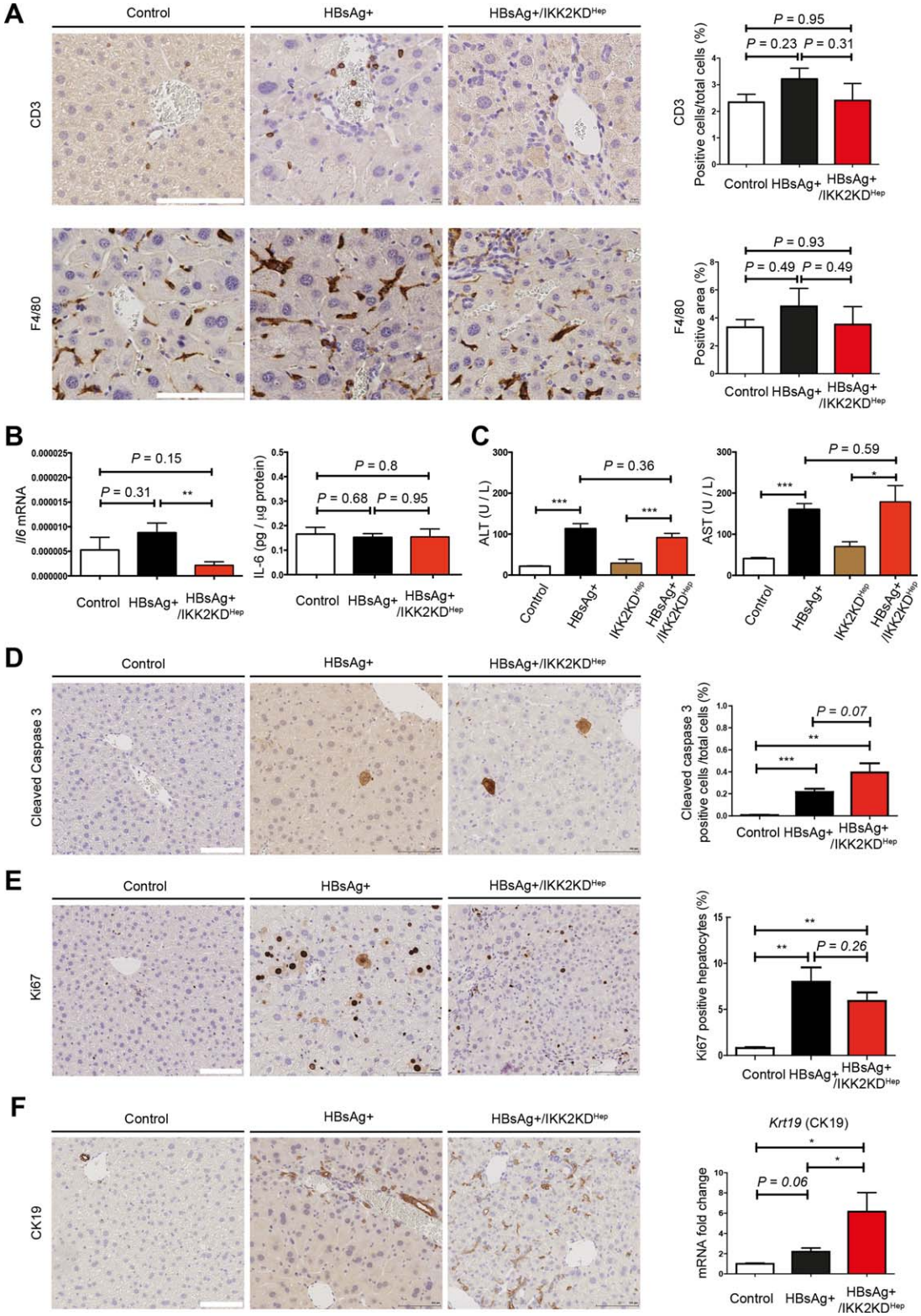

FIG. 3. Inhibition of canonical NF-κB signaling in HBsAg$^+$ transgenic mice disrupts compensatory liver regeneration with concomitant occurrence of ductular reactions. (A) IHC for CD3 (T lymphocytes) and F4/80 (macrophages/Kupffer cells) in livers of 20-week-old mice (bar = 100 μm) and morphometric quantification of CD3-positive lymphocytes (percent of total cells) and F4/80-positive area (control n = 3, HBsAg$^+$ and HBsAg$^+$/IKK2KD^{Hep} n = 7). (B) mRNA levels for *Il6* by qPCR shown as fold change (control n = 4, HBsAg$^+$ and HBsAg+/IKK2KD^{Hep} n = 8). IL-6 protein level from liver lysates determined by enzyme-linked immunosorbent assay (n = 7-10). (C) Serum alanine aminotransferase and aspartate aminotransferase levels of 20-week-old mice (control n = 26, HBsAg$^+$ n = 28, IKK2KD^{Hep} n = 10, and HBsAg$^+$/IKK2KD^{Hep} n = 8). (D) IHC for caspase-3 cleavage (bar = 100 μm) with morphometric quantification (n = 4-7). (E) IHC for Ki67 expression (bar = 100 μm) with morphometric quantification for Ki67-positive hepatocytes (n = 4-7). (F) Ductular reaction, characterized by IHC for cytokeratin 19 and relative mRNA expression of cytokeratin 19 determined by qPCR (control n = 3, HBsAg$^+$ n = 6, HBsAg$^+$/IKK2KD^{Hep} n = 5). Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK19, cytokeratin 19.

3B). In fact, *Il6* mRNA expression was actually significantly reduced in HBsAg^{+}/IKK2KD^{Hep} livers compared to HBsAg^{+} mice. Moreover, NF-κB inhibition in HBsAg^{+} livers did not further increase transaminase levels (Fig. 3C). These data argue against an increased necroinflammatory environment as explanation for the drastically increased HCC incidence in HBsAg^{+}/IKK2KD^{Hep} transgenic mice, which is in contrast to the diethylnitrosamine or the hepatocyte-specific NEMO knockout model.[5,33]

Hepatocyte apoptosis, as analyzed by cleaved caspase-3 immunohistochemistry (IHC) at 20 weeks, was frequently detected in HBsAg^{+} transgenic mice and slightly increased after inhibiting canonical NF-κB (Fig. 3D). IHC for Ki67 showed strong hepatocyte proliferation in HBsAg^{+} single-transgenic mice, indirectly indicating elevated hepatocyte apoptosis due to HBsAg expression (Fig. 3E). Surprisingly, proliferation of hepatocytes was not further increased in HBsAg^{+}/IKK2KD^{Hep} animals (Fig. 3E).

Hematoxylin and eosin staining revealed an increase in small cells in HBsAg^{+}/IKK2KD^{Hep} livers which were negative for immune cell markers and positive for the biliary linage marker cytokeratin 19 (Fig. 3F), indicating a ductular reaction (i.e., oval cell response).

INHIBITION OF CANONICAL NF-κB SIGNALING LEADS TO A DOWN-REGULATION OF THE UPR MASTER REGULATOR BINDING IMMUNOGLOBULIN PROTEIN/78-KDA GLUCOSE-REGULATED PROTEIN AND ENHANCED ER STRESS

HBsAg was distributed unevenly throughout livers and in the ER of hepatocytes when the canonical NF-κB pathway was blocked (Fig. 4A,B). However, serum HBsAg was unchanged in HBsAg^{+}/IKK2KD^{Hep} compared to HBsAg^{+} single-transgenic mice (Fig. 4C). This suggests that inhibition of canonical NF-κB signaling influences HBsAg protein processing, possibly due to increased ER stress, as overproduction of the HBV large envelope polypeptide in HBsAg transgenic mice was previously shown to lead to accumulation of filamentous HBV surface particles within the ER of hepatocytes.[13]

We therefore measured mRNA expression of genes known to be involved in ER stress, inflammation, and DNA damage. In 20-week-old HBsAg^{+}/IKK2KD^{Hep} mice, expression of genes associated with ER stress and the UPR were altered when compared to HBsAg^{+} single-transgenic and littermate control mice (Fig. 4D; Supporting Fig. S4A). Among the most significantly down-regulated genes compared to HBsAg^{+} was binding immunoglobulin protein (BiP)/78-kDa glucose-regulated protein, a molecular chaperone of the HSP70 family that acts as a master regulator of newly synthesized protein folding (Fig. 4E).[19]

When aggregated proteins accumulate in the ER, BiP determines whether unfolded or misfolded proteins should be targeted to ER stress-associated degradation and to promote cell survival or to direct cells toward apoptosis.[34] Consistent with our gene expression analysis (Fig. 4E), we observed decreased BiP protein expression by both western blotting and IHC after NF-κB inhibition in HBsAg^{+}/IKK2KD^{Hep} mice compared to single-transgenic HBsAg^{+} or control mice (Fig. 4E; Supporting Fig. S5D).

These data suggest that inhibiting canonical NF-κB signaling in hepatocytes in the context of HBsAg expression leads to decreased hepatic BiP and thus disrupted ER stress sensing. Promoter analysis using JASPAR predicted with 85% probability that the promoter region of human and mouse *HSPA5/hspa5* genes encoding BiP lacked any NF-κB-binding site (Supporting Fig. S5E; Supporting Table S2). Furthermore, BiP was shown not to be a direct NF-κB target gene,[27] and normal expression was detected by IHC after NF-κB inhibition in IKK2KD^{Hep} single-transgenic mice (Supporting Fig. S5A-C), arguing for an indirect effect of NF-κB signaling on BiP expression during prolonged ER stress.

ER transmembrane signal transducers such as activating transcription factor 6 (ATF6) and PERK are released from BiP upon accumulation of unfolded proteins. Free ATF6 translocates from the ER to the Golgi, where it is proteolytically processed.[35] PERK dissociation from BiP triggers activation of eIF2 by phosphorylation and ATF4 expression. These signaling pathways regulate ER stress-associated gene expression, including the cell death regulator CHOP.[20]

We consistently observed ER stress signaling by significantly increased processed ATF6 protein, enhanced eIF2 phosphorylation, as well as elevated ATF4 and CHOP expression in HBsAg^{+}/IKK2KD^{Hep} mice (Fig. 4F; Supporting Fig. S5F).

Therefore, we conclude that canonical NF-κB plays an important role in controlling the UPR. Inhibition of NF-κB in mice expressing HBsAgs at high levels

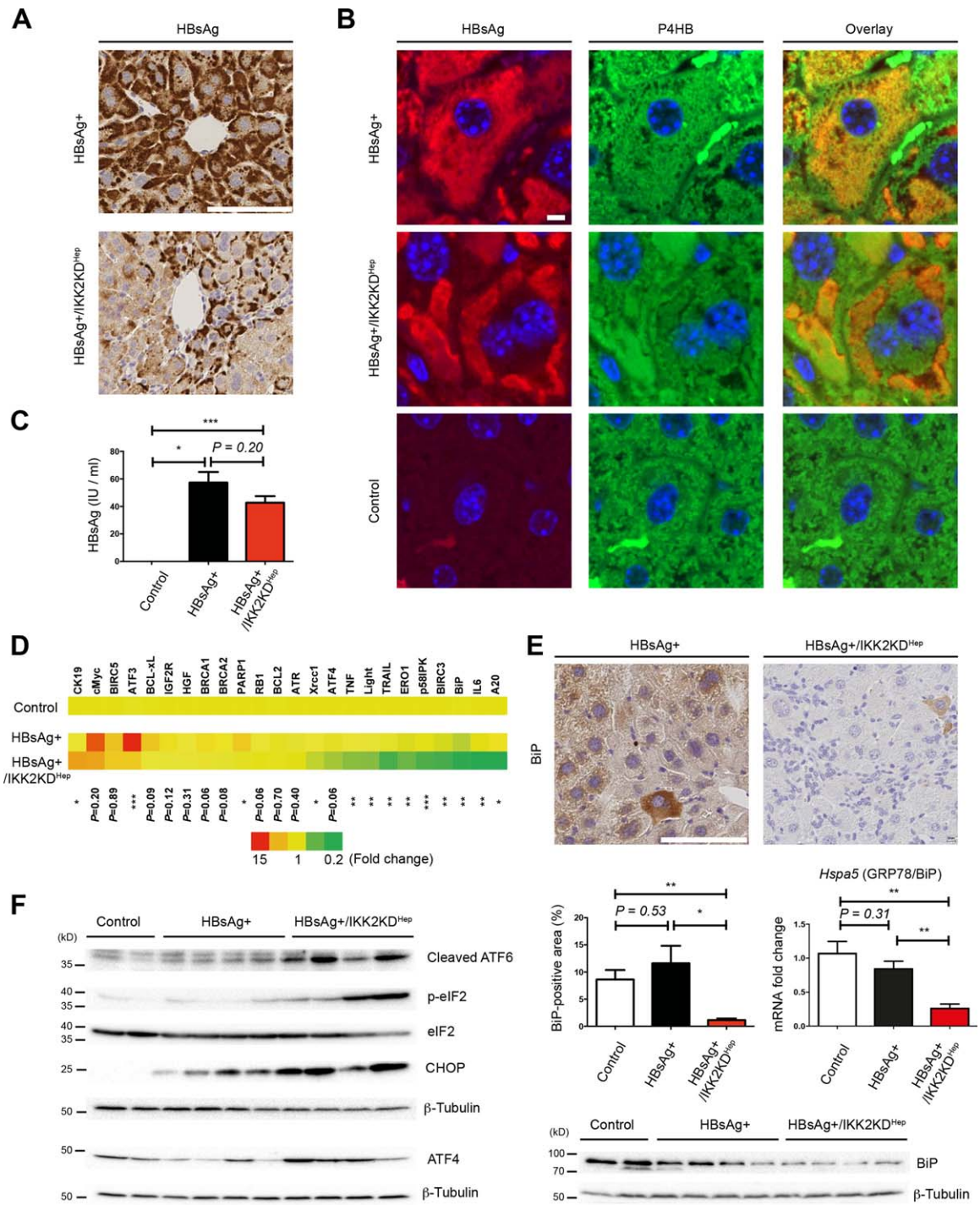

FIG. 4. Inhibition of NF-κB signaling leads to prolonged ER stress signaling and reduced expression of the UPR regulator BiP. (A) Representative IHC for HBsAg in $HBsAg^{+}$ and $HBsAg^{+}$/$IKK2KD^{Hep}$ livers at 20 weeks of age (bar = 100 μm). (B) Representative immunofluorescence staining of whole liver tissue of $HBsAg^{+}$ and $HBsAg^{+}$/$IKK2KD^{Hep}$ mice showing HBsAg accumulation (red) in the ER of hepatocytes labeled with the ER marker P4HB (green). 4′,6-Diamidino-2-phenylindole (blue) shows nuclei of cells, and overlay displays colocalization (bar = 10 μm). (C) Quantitative serum analysis of HBsAg levels in $HBsAg^{+}$ versus $HBsAg^{+}$/$IKK2KD^{Hep}$ mice (control n = 3, $HBsAg^{+}$ n = 8, and $HBsAg^{+}$/$IKK2KD^{Hep}$ n = 5). (D) mRNA expression levels of genes involved in apoptosis, DNA damage, and UPR control; means of fold change (of n = 3-6 animals per group) were summarized in a heat map. Statistical analysis is shown between $HBsAg^{+}$ and HBsAg+/$IKK2KD^{Hep}$. (E) IHC for BiP expression in $HBsAg^{+}$ and $HBsAg^{+}$/$IKK2KD^{Hep}$ livers (bar = 100 μm) with morphometric quantification of IHC for BiP (left graph, control n = 3, $HBsAg^{+}$ and $HBsAg^{+}$/$IKK2KD^{Hep}$ n = 6), mRNA expression of BiP determined by qPCR (right graph, n = 6), and western blot analysis of whole-liver extracts using antibodies against BiP and β-tubulin as loading control. (F) Western blot analysis of factors involved in the UPR, using antibodies against ATF6, eIF2, phospho-eIF2, ATF4, CHOP, and β-tubulin as a loading control (for quantification see Supporting Figs. S5 and S6).

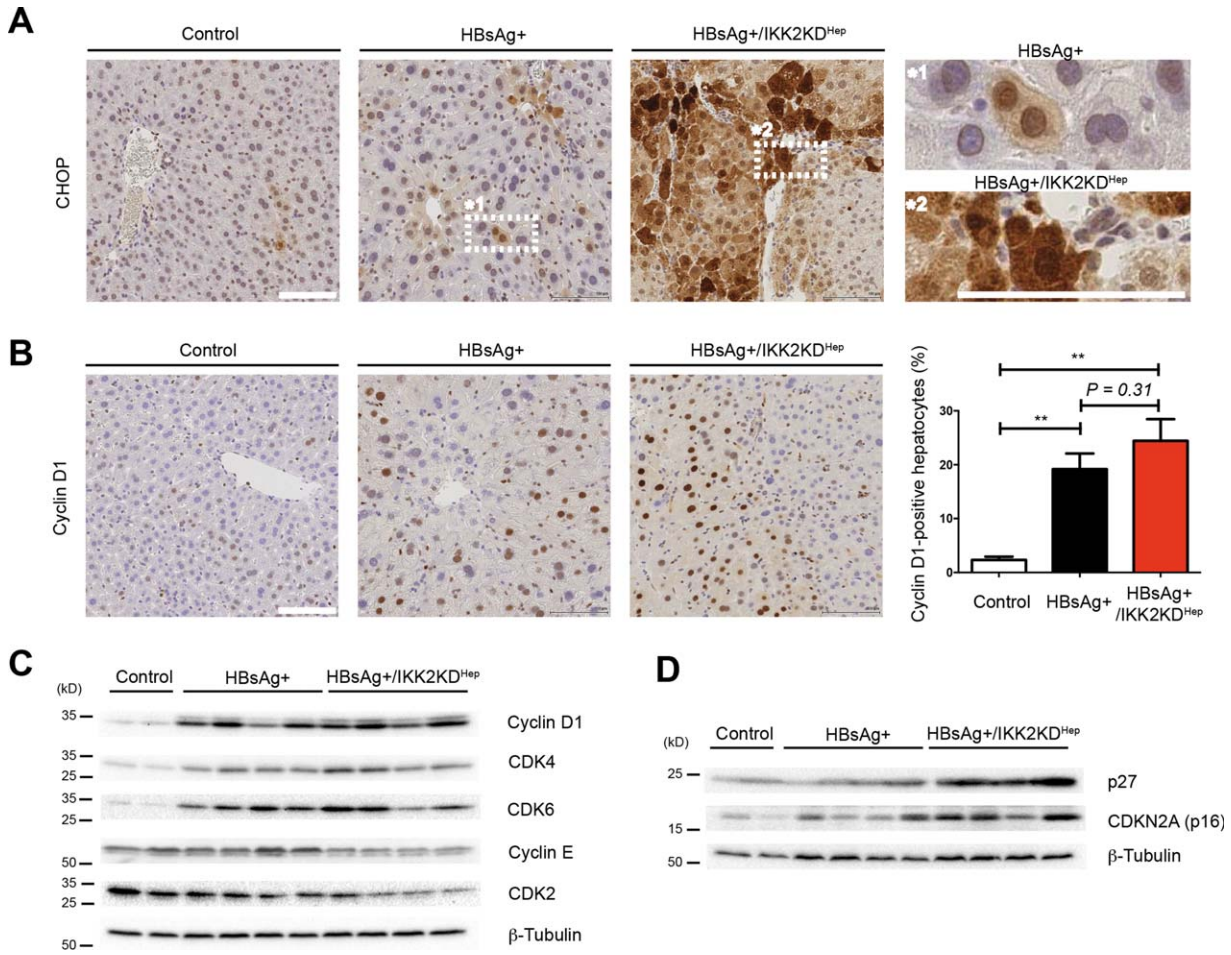

FIG. 5. Hepatocytes of HBsAg$^+$/IKK2KD^{Hep} mice display CHOP overexpression and G_1/S-phase cell cycle arrest. (A) Overexpression of CHOP in HBsAg$^+$/IKK2KD^{Hep} livers shown by IHC (bar = 100 μm). (B) IHC analysis for cyclin D1 shows expression in hepatocytes (bar = 100 μm) and morphometric quantification of cyclin D1$^+$ hepatocyte nuclei (n = 4-6). (C) Expression of factors involved in cell cycle control analyzed by western blot in livers using antibodies against cyclin D1, CDK4, CDK6, cyclin E1, and CDK2. (D) Expression of cell cycle inhibitors p16, p27, and β-tubulin as a loading control in western blot (for quantification, see Supporting Fig. S6).

leads to improper expression of the ER stress master regulator BiP and uncontrolled activation of the UPR signaling pathways.

HBsAg$^+$/IKK2KD^{Hep} HEPATOCYTES DISPLAY CHOP OVEREXPRESSION AND G_1/S-PHASE CELL CYCLE ARREST

We observed excessively increased CHOP expression in HBsAg$^+$/IKK2KD^{Hep} mice by IHC analyses (Fig. 5A). Notably, NF-κB inhibition alone in IKK2KD^{Hep} single-transgenic control mice does not significantly change CHOP expression (Supporting Fig. S6A-C). CHOP not only is a key regulator of ER stress-associated apoptosis but also attenuates cellular proliferation.[36,37] Because HBsAg$^+$/IKK2KD^{Hep} hepatocytes showed an uncontrolled UPR and lacked any further increase in ki67$^+$ (Fig. 3E; Supporting Fig. S6D), did not show significant increased apoptosis (Fig. 3D), yet did show the emergence of ductular reactions (Fig. 3F), we asked whether there is a defect in cell cycle progression. Both HBsAg$^+$ and HBsAg$^+$/IKK2KD^{Hep} mice showed increased cyclin D1 expression in hepatocytes at 20 weeks as shown by IHC and western blot analyses (Fig. 5B,C). Conversely, expression of cyclin E and CDK2 was decreased in

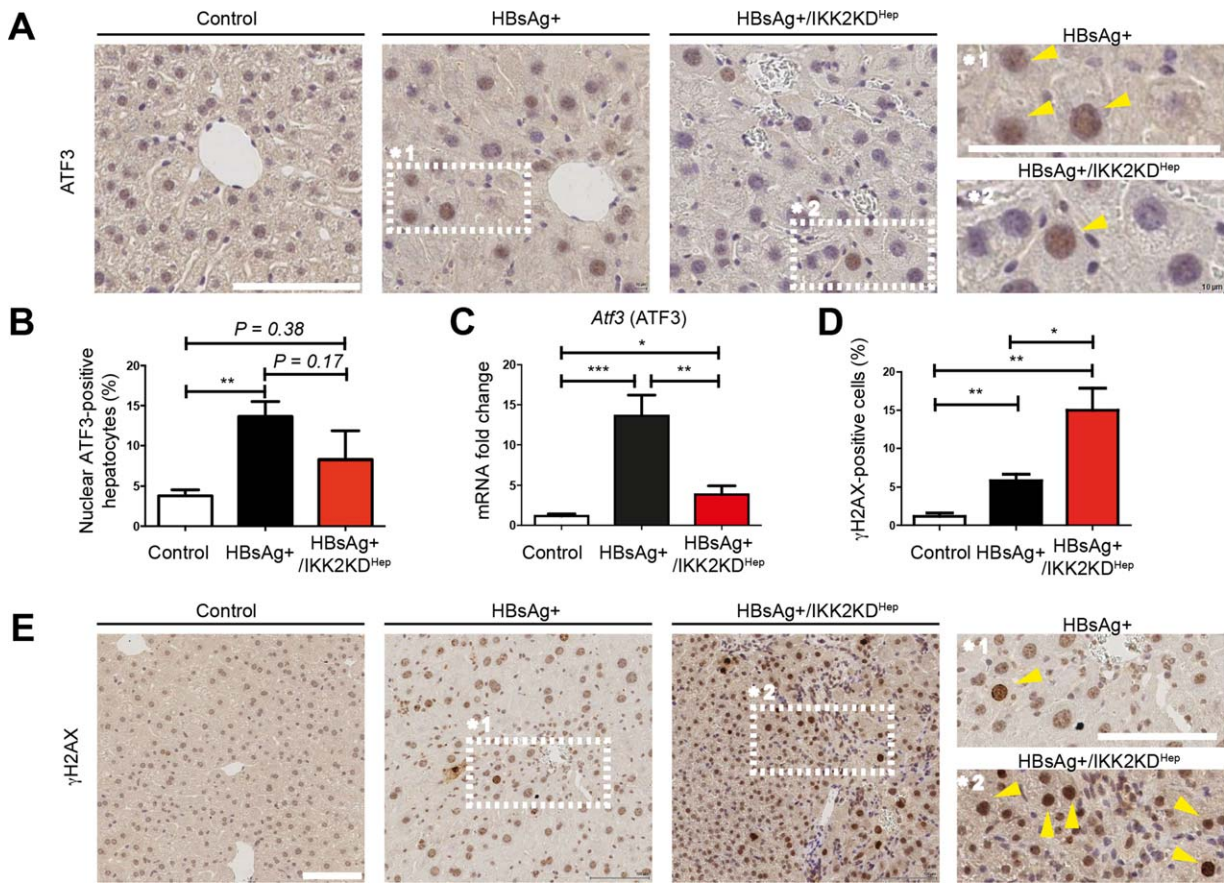

FIG. 6. Attenuated ATF3 expression and accumulation of DNA damage due to uncontrolled ER stress. (A) IHC for ATF3 shows reduced nuclear staining in hepatocytes after NF-κB inhibition (bar = 100 μm). (B) Morphometric quantification of ATF3$^+$ hepatocyte nuclei, and (C) relative mRNA expression of ATF3 determined by qPCR (control n = 3, HBsAg$^+$ and HBsAg$^+$/IKK2KD^{Hep} n = 6). (D) Morphometric quantification of nuclear positivity for the DNA damage marker γH2AX (n = 4), and (E) IHC for the DNA-damage marker γH2AX and magnifications displaying positive hepatocytes (arrowheads; bar = 100 μm).

HBsAg$^+$/IKK2KD^{Hep} livers compared to HBsAg$^+$ (Fig. 5C; Supporting Fig. S6E), indicative of a late G_1-phase cell cycle arrest.

Because overexpression of CHOP can lead to G_1/S-phase cell cycle arrest,[37] we analyzed expression of cell cycle inhibitors. We found elevated p27/Kip1 and p16/CDKNA2 in HBsAg$^+$/IKK2KD^{Hep} livers compared to HBsAg$^+$ or control (Fig. 5D; Supporting Fig. S6E), resembling well-known cell cycle inhibitors activated by cellular stress-blocking cyclin D/CDK4 and CDK2 activity.[38,39] These data suggest that inhibition of canonical NF-κB signaling in hepatocytes expressing high levels of HBsAg leads to CHOP overexpression and G_1/S-phase cell cycle arrest, indicating severe cellular stress and inadequate liver regeneration in hepatocytes due to uncontrolled ER stress.

PROLONGED ER STRESS IN HEPATOCYTES PROMOTES ACCUMULATION OF DNA DAMAGE AND INCREASED HCC INCIDENCE

Even though HBsAg$^+$/IKK2KD^{Hep} hepatocytes show prolonged, uncontrolled ER stress with accumulation of CHOP and cell cycle arrest, ATF3 expression and nuclear ATF3-positive hepatocytes were reduced compared to HBsAg$^+$ mice (Fig. 6A-C). ATF3 is an adaptive response gene induced by various cellular stress stimuli to activate or repress gene expression and to act as a tumor suppressor by promoting cell death and suppressing cell cycle progression. As a consequence of this uncontrolled cellular stress response

after NF-κB inhibition, high levels of DNA damage in hepatocytes were already detected at 20 weeks of age in $HBsAg^{+}$/$IKK2KD^{Hep}$ mice, as shown by serine-139-phosphorylated γH2AX levels (Fig. 6D,E).

We conclude that, in the case of HBsAg-driven HCC, canonical NF-κB signaling plays an indispensable role in properly controlling the UPR. The absence of canonical NF-κB signaling leads to a prolonged, uncontrolled UPR with loss of the UPR master regulator BiP, overexpression of CHOP, and cell cycle arrest, indicative of increased cellular stress. This is accompanied by accumulation of DNA damage and subsequent 100% HCC incidence.

Discussion

Here, we identify a novel role of NF-κB signaling in controlling ER stress response, which acts as a tumor-suppressor in HBV protein-driven HCC development. We found that NF-κB is essential for proper UPR control and BiP expression. Moreover, loss of NF-κB causes disrupted UPR with CHOP overexpression, induction of cell cycle arrest in hepatocytes, and occurrence of ductular reactions. Finally, due to prolonged ER stress, DNA damage accumulates in hepatocytes, culminating in markedly enhanced HBsAg-driven hepatocellular carcinogenesis.

Human HCC is typically preceded by chronic inflammation, cell death, and compensatory liver proliferation; however, in CHB infection HCC can occur in the absence of cirrhosis and severe necroinflammation. In so-called inactive CHB patients HCCs still occur, despite efficient suppression of HBV viremia and necroinflammation by nucleos(t)ide analogue therapy, particularly in patients with high HBsAg levels.[9-11] In addition to HBV DNA integration and effects of the enigmatic HBV x-protein, ground glass hepatocytes, containing accumulated HBV surface proteins, as well as preS1/2 mutants, C-terminally truncated M protein and overexpression of HBV large surface proteins are found in chronically HBV-infected patients and exert oncogenic functions.[15-17] Most importantly, observations from CHB patients under conditions of immune suppression and in HBV transgenic mouse models suggest that in the absence of adaptive immune responses and NF-κB signaling, cellular stress induced by HBV may also lead to development of liver disease and HCC.[21-23]

In the case of the HBsAg-driven liver cancer model, where HBsAg is expressed as a transgene and is not detected by the host immune system due to central immune tolerance,[6] we did not detect secondary inflammation, increased transaminases, or increased IL6 levels after NF-κB inhibition (Fig. 3), which was reported in other models showing an antitumorigenic role for NF-κB signaling using hepatocyte-specific deletion of NEMO or IKK2.[5,33]

Almost three dacades ago, studies demonstrated the oncogenic potential of overexpressed HBV envelope polypeptides and accumulation of filamentous HBsAg particles within the ER as a potential mechanism.[13,14] Accumulation of unfolded/misfolded proteins activates intracellular signaling pathways associated with ER stress. This is buffered by activation of the UPR, a homeostatic signaling network responsible for recovering ER function, or if this fails, triggering apoptosis.[34]

In ER stress sensing and UPR, BiP is the central regulator; and in the current study, we demonstrate that inhibition of canonical NF-κB signaling leads to inadequate expression of BiP in the HBsAg model (Fig. 4E; Supporting Fig. S5). It has been reported that NF-κB does not induce BiP expression[27]; however, another study suggested that the UPR induces RelA/p65 binding to the BiP promoter region.[28] We did not observe any NF-κB-binding site in the BiP promoter, and BiP is not directly down-regulated due to loss of NF-κB signaling as homeostatic BiP expression was not altered in $IKK2KD^{Hep}$ control animals, indicating indirect regulation of BiP by NF-κB. We clearly demonstrate that NF-κB signaling is required for UPR control and sustained BiP expression. Moreover, inhibition of canonical NF-κB signaling not only attenuates BiP expression but also leads to overexpression of CHOP (growth arrest and DNA damage-inducible gene 153) in association with activation of the ATF6 and eIF2/ATF4 pathways in $HBsAg^{+}$ mice, thus generating a sustained ER stress response due to HBsAg expression. Consistent with our results, it has been shown that BiP overexpression down-regulates CHOP[40]; vice versa, down-regulation of BiP is associated with processing of ATF6 (activation) and consequent overexpression of ATF4 and CHOP. Overexpression of CHOP in hepatocytes after NF-κB inhibition was found in almost all hepatocytes; however, no concomitant significant increase in apoptotic hepatocytes was detectable (Fig. 4).

CHOP is a key regulator of ER stress-associated apoptosis and is associated with liver cancer development; however, its precise role is not yet clear.[41] CHOP expression was reported to be up-regulated in human HCC. In addition, complete deletion of *Ddit3*

(encoding CHOP) in mice confers resistance to chemical hepatocarcinogenesis by attenuating both apoptosis and cellular proliferation.[36] Furthermore, CHOP overexpression blocks cells from progressing from the G_1 to the S phase by dimerizing with other CCAAT/enhancer binding proteins and being directed away from "classical" CCAAT/enhancer binding protein sites.[37]

Inadequate liver regeneration was associated with sustained CHOP expression and, in the case of NF-κB inhibition in our $HBsAg^+$ transgenic model, was accompanied by overexpression of p27 (Fig. 5). This is consistent with the known activation of cell cycle inhibitors due to cellular stressors, such as prolonged ER stress. The cell cycle inhibitors INK4α (CDKNA2/p16) and p27 (Kip1) block cyclin D/CDK4 and CDK2 activity,[38,39] which may explain the inability of hepatocytes to initiate proper compensatory proliferation as well as the occurrence of ductular reactions, suggesting inadequate liver regeneration. Intrahepatic ductular reactions due to impaired hepatocyte proliferation have been described. Oval cells were initially believed to serve as a substitute progenitor compartment for both cholangiocytes and hepatocytes if acute or chronic liver injury compromises self-duplication of the mature epithelial cells.[42] However, recent data demonstrate that the adult liver progenitor compartment within biliary compartment-derived ductular reactions does not function to replenish a significant proportion of hepatocytes, and ductular cells are not the main source of malignant transformation in HCC[43,44] but rather indicate severe liver damage.

Consistent with a disrupted UPR response and loss of ER stress control after NF-κB inhibition in the HBsAg model, expression of the general cellular stress response factor ATF3 was decreased in comparison to $HBsAg^+$ mice (Fig. 6). ATF3, an adaptive-response gene induced by cellular stress, represses cyclinD1 and CHOP expression. Due to its tumor-suppressor functions, attenuated ATF3 is likely to contribute to increased HCC development.[45] Conversely, overexpression of ATF3 in tumor tissue was reported to contribute to chemoresistance and invasive growths.[46]

In our model we propose that lack of canonical NF-κB signaling disrupts cellular stress responses that would be initiated by ER stress and aggravates pathology due to enhanced cellular stress. Cellular stressors such as prolonged ER stress are known to increase the sporadic occurrence of DNA damage. Damage that occurs within stressed cells must be tightly controlled to prevent either loss of function or the clonal persistence of oncogenic mutations that increase the risk of tumorigenesis.[47] This is consistent with our finding of significantly increased DNA damage already at 20 weeks of age due to loss of UPR control after NF-κB inhibition in $HBsAg^+$ mice. These events finally culminate in a 100% incidence of hepatocellular carcinogenesis.

However, the mechanism identified here may not be specific to HBV as ER stress also has been shown to be potentially oncogenic in HCV and nonalcoholic steatohepatitis.[29,48,49] Nevertheless, HCV-infected human livers usually show no apparent induction of UPR-responsive genes, which is in line with CHB patients.[50] As in $HBsAg^+$ mice, we did not detect significant CHOP up-regulation in human samples (data not shown) as ER stress is normally tightly controlled. This underlines the importance of intact NF-κB signaling in ER stress.

Further studies are needed to determine whether these mechanisms are applicable only to HBsAg-driven or also to general ER stress-associated tumor models. Nevertheless, our study highlights a critical role of NF-κB for UPR control in liver cancer development.

Thus, any treatment inhibiting NF-κB signaling in patients harboring CHB is best avoided, and in general, previously proposed therapeutic approaches targeting NF-κB signaling in HCC development may not be applicable in the context of CHB infection and ER stress conditions.

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Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28435/suppinfo.